Synopsis of the thesis on

Exploring *Chlamydia trachomatis* Immunopathogenesis

To be submitted to

For the degree of

Doctor of Philosophy in Biotechnology

by

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Respected Sir,

Kindly accept the synopsis of my Ph.D work entitled - "**Exploring *Chlamydia trachomatis* immuno-pathogenesis**". My date of registration was 07.01.2017 and registration number is UIAR/10839.

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Abstract

Chlamydia trachomatis is an obligate intracellular bacteria majorly infecting the female genital tract. The infection elicits an inflammatory response activating IFNγ production from $CD4^+$ T cells. IFNγ clears off the pathogen in epithelial cells and macrophages. However, during chronic infection Th_1 generation is impaired due to low expression of CD40 on macrophages and epithelial cells. In addition, chronic *C. trachomatis* induces PDL1 expression on macrophages facilitating Treg generation. Chronic infection also skews myelopoiesis to generate M_2 macrophages which harbor higher bacteria compared to M_0 and M_1 macrophages. The generation of M_2 and Treg during the chronic infection fails to clear the pathogen leading to the persistence of the pathogen. On secondary infection, the Tregs are generated preferentially compared to Th_1 cells. T cell proliferation is also poor owing to the increased expression of *KLRG3*, *PD1*, and *CTLA4*. Due to the absence of M_1 macrophages and Th_1 cells, *C. trachomatis* continues to grow in the genital tract. Chronic infection also induces proliferation of the epithelial cells which resisted TNFα and H_2O_2 induced cell death. The effect is more pronounced in presence of HPV16 E6-E7 proteins in mice. In mice, the presence of *C. trachomatis* and HPV16 E6-E7 generated epithelial cells demonstrate extended proliferation in the in the *in vitro* cultures. In conclusion, chronic *C. trachomatis* induces immunosuppression in macrophages which is translated to defective T cells and this chronic inflammation along with cellular changes bought by the pathogens might be inducing oncogenic-like properties in the epithelial cells.

Keywords: *Chlamydia trachomatis*, Macrophage polarisation, CD40, IFNγ, E6-E7, cervical cancer

Contents

Abstract **iv**

Content **v**

1 Introduction **1**

1.0.1 T cell activation 4

1.1 Review of literature 6

1.2 Materials and Methods 8

1.2.1 Mouse model of genital *C. trachomatis* infection 8

1.2.2 Bacteria and Bacterial lysate 9

1.2.3 *C. trachomatis* infection and maintenance 9

1.2.4 DNA isolation and qPCR for bacterial burden 9

1.2.5 Other Techniques used in the study 10

1.3 Results and Disucssion 11

1.3.1 Live *C. trachomatis* induces M_2 macrophages 11

1.3.2 CD40 activation during *C. trachomatis* infection promote anti-inflammatory phenotype 12

1.3.3 Chronic *C.trachomatis* induces M_2 macrophages 15

1.3.4 Acute and chronic genital *C.trachomatis* infection show differential immune response 16

1.3.5 *C. trachomatis* infection alters cell cycle in epithelial cells . . . 23

1.3.6 *C. trachomatis* and HPV co-infection induce tumorigenic changes in mouse cervical tissue 27

1.4 Conclusion 28

References **31**

List of Publications **35**

Chapter 1

Introduction

Chlamydia trachomatis and its etiology

Chlamydia trachomatis belongs to Chlamydiaceae family which consists of two genus *Chlamydophilia* and *Chlamydia*. *Chlamydia* has majorly three species *C. trachomaits*, *C. suis* and *C. muridarum*. Chlamydiae are obligate intracellular bacteria. Typically they show similar kinds of pathologies, thus studies with *C. muridarum* infection in mice models are more prevalent to understand the *C. trachomaits* infection. Despite various models, the pathology at the genital tract using *C. muridarum* is difficult to study and does not justify its role in genital infections. Thus we have used the *C. trachomaits* to study chronic genital infection-mediated immunopathologies. *C. trachomaits* has a genome size of 1.039Mb and a plasmid of 7.4kb. Despite varying host ranges and routes of infection, the chlamydial genomes are remarkably similar[1]. These plasmids might show variation and encode for 8 genes. This plasmid encodes for gene products that play a role in the intracellular growth of the organism[2]. Genital infection of *Chlamydia trachomatis* is one of the most common sexually transmitted diseases worldwide. Disease in women leads to severe outcomes such as pelvic inflammatory disease, ectopic pregnancy, and infertility. According to the global burden of disease report by WHO, it is the 10^{th} most common infection with an estimated 131 million new infections yearly. Center for Disease Control (CDC) estimates that four million chlamydial infections occurred in 2018. Most of the *C. trachomatis* infections are asymptomatic and resolve themselves. Asymptomatic infection can also have damaging effects on the reproductive system. In some cases, symptoms might include discharge and a burning feeling while urinating. Apart from genital infections, *C. trachomatis* can also infect the eye causing trachoma. It is responsible for the blindness or visual impairment of in about 1.9 million people in 44 countries. Prevalence of *C. trachomatis* infections in India ranges from 0.4% to 29% with high incidences among commercial sex workers[3].

Distribution of the *C. trachomatis* related deaths in India are plotted in the below graph (Fig.1.1).

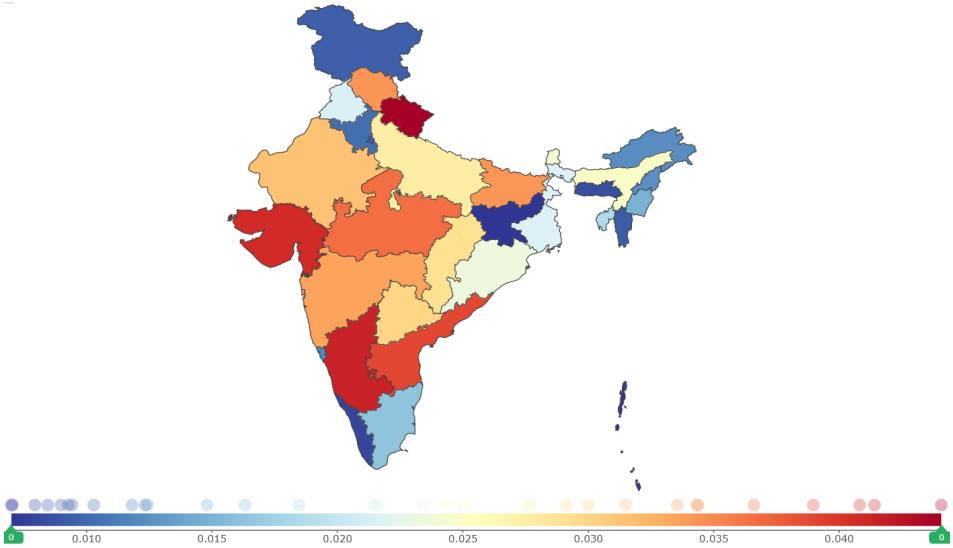


Figure 1.1: Distribution of *C. trachomatis* related death in India in 2019[4]

In addition to severe reproductive complications, *C. trachomatis* infection has also been proposed as a possible risk factor of *Human papillomavirus* (HPV) infection, known to cause cervical cancer. Chronic infection of *C. trachomatis* is consistently found with HPV facilitating or promoting the infection. Early stage infection of *C. trachomatis* is mostly asymptomatic and resolves, but repeated exposure or the persistent infection spreads to fallopian tubes. Genital *C. trachomatis* infection follows fallopian tube scarring leading to infertility or ectopic pregnancy. History of infection with *C. trachomatis* has also been linked to the development of cervical cancer. Even though researchers for a very long time have been indicating co-infection or co-occurrence of HPV and *C. trachomatis* in patients suffering from sexually transmitted diseases (STDs), but their molecular architecture and significance to the host pathology have not been explored. The co-infection of HPV and *C. trachomatis* have to be considered in the context of cervical cancer. Immune response in the human body is a dynamic and continuous process tackling external and internal insults. Immunity is the second most complex system in the body next to the nervous system. It involves various organs working coordinately for host protection against pathogens and internal mutations. In the process of pathogenic elimination, it must avoid responses that damage self-tissue; thus, a control mechanism is in place with the help of certain immune cells. The major part of this mechanism is the recognition of distinct structures of pathogens and altered protein structures. Immune activation is a coordinated event between many cells and tissues located distantly in the body. Majorly, bone marrow and thymus are the primary lymphoid organs that produce immune cells. Secondary

lymphoid organs include lymph nodes, spleen, and tonsils where the cells generated at the primary lymphoid organs get activated and replicate for amplified immune response. The cells of the immune system can broadly be divided into innate and adaptive immune cells. Innate immune cells are the first line of defense against insults by engulfing via phagocytosis or endocytosis and inducing inflammatory response to activate adaptive immune cells. Major cells involved in the innate immune response are macrophages, neutrophils, dendritic cells, mast cells, basophils, eosinophils and natural killer cells. Cells like macrophages and dendritic cells process the engulfed pathogen and present via MHC-I or MHC-II (antigen presenting cells) to adaptive immune cells to enable them for specific immune responses. They also secrete cytokines which can directly affect the pathogens or pathogen-containing cells. Adaptive immune cells are effective and specific in eliminating the pathogen. T cells and B cells are involved in the adaptive immune response which is further divided into various types based on their functions. In overview, T helper cells help in the activation of cytotoxic T cells and B cells with their secreted cytokines; cytotoxic T cells secrete cytotoxic proteins inducing cell death in pathogen containing/altered cells. B cells secrete antibodies, which bind to antigens disrupting pathogenic insults and enabling higher pathogenic uptake.

Immune responses in mucosal tissues are governed by the nature of the antigen, the type of APC's involved, and the local environment. Epithelial cells take up antigens or infected epithelial cells present the antigens to T cells at the inductive sites. APCs shuttling at the tissue, present processed antigens to $CD4^+$ and $CD8^+$ T cells. These sensitized immune cells, leave the initial site with antigen, and transit through the lymph and blood to lymph nodes where they activate naïve cells to amplify the immune response[5].

Innate immune cells detect microorganisms by their unique molecular structures through pathogen recognition receptors (PRRs). There are several PRRs including TLRs and NLRs which are explained in detail in a later section on innate immune cells, which upon activation produce inflammatory cytokines, including TNF, IL-1β and IL-6 coordinating local and systemic inflammatory responses. When a pathogen is encountered by a dendritic cell, it is taken up by phagocytosis and is digested in the phagosome. The resulting protein constituents called antigenic peptides are processed and presented at the cell surface by MHC I or II. TLRs activation also induces macrophages to produce anti-microbial proteins and peptides. These activated macrophages with microbial antigens travel to adjacent lymph nodes or spleen. The antigen is presented to lymphocytes enabling them to differentiate into several types of effector cells depending on the pathogen. Lymphocytes could be T cells or B cells. Conventionally, T cells with $\alpha\beta$ CD3 chains are marked by $CD4^+$ (Th cells) and $CD8^+$ (Tc cells) co-expression. Through extensive investigations, it is often observed that, although innate

immune cells are crucial for controlling infection, it is insufficient for pathogen clearance. The immune activation also shows negative effects affecting tissue physiology and may direct tissue damage resulting in immunopathology. It is crucial for the host to limit the immunopathology in parallel with pathogen clearance. The optimum balance of sensitivity and intensity of immune activation. The balance is difficult to achieve, as immune responses need to vary in intensity and duration depending on the infection pathogen and intensity of the infection. Thus, the directed immune response achieved by effector lymphocytes is crucial for the appropriate protective immunity [5].

Immunologic responses process memory and recall upon secondary infection to counteract the pathogen efficiently and precisely. However, some allergens, non-pathogenic microorganisms, chronically infected cells, and tumor cells fail to provoke effective immune responses despite their ability to demonstrate antigenicity. This is to protect the host from constitutive immune-mediated damage. The implicit reason why the outcomes vary greatly and damage is controlled is by the natural immunoregulatory process actively suppressing the immune response to constant antigenic stimuli. However, weak or ineffective immune responses play a pathogenic role by allowing chronic infections like tuberculosis, chlamydiosis, leishmaniasis, HIV, etc, to persist in immunotolerance conditions. The tendency to provoke immunotolerance rather than immune activation at the mucosal surfaces challenges the notion of self-versus and non-self-antigens in the adaptive immune system. Some pathogens also manipulate adaptive immunity by inducing immunoregulatory mechanisms to persist for long periods in the host. Through this thesis work, we emphasize mechanisms of such immunoregulatory networks suppressing host immunity and promoting tolerance leading to pathogen persistence in the host.

1.0.1 T cell activation

Upon activation, various cytokines are secreted from the APCs which will activate, recruit and amplify other immune cells. Upon contact with T cells, APCs prime T cells with cognate interactions between MHC and TCR. Apart from activating T cells in proximity, APCs travel to the secondary lymphoid organs, where they prime T cells. Antigen recognition by the T cell activates TCR signaling which triggers a series of generic, proliferative, differentiation and biochemical changes which continue from minutes to days leading to pathogen-specific clonal expansion. Along with the expansion, differentiation of naïve T cells to effector T cells happens with the help of cytokines and APC-T cell cognate interaction[6,7,8].

The activation of T cells happens in three phases:

1. Priming and expansion

2. Resolution and contraction and
3. Memory formation

The cytokines and other proteins secreted from the T cells and innate immune cells act on cells to kill the pathogens. Key executioner cytokines that mediate bacterial killing are TNFα, IFNγ, perforin, and granzyme.

Cytokines influencing T cell differentiation	T cell	Transcription factor	T cell cytokine
IL-12, IL-18	Th_1	T-bet, STAT1/4	IFNγ
IL-6, TGFβ, IL-1β	Th_{17}	RORγt, STAT3	IL-17, IL-21, IL-22
IL-6, TNFα	Th_{22}	Ahr	IL-22
IL-4,	Th_2	Gata3, STAT5/6	IL-4, IL-5, IL-13, IL-10
IL-10, TGFβ	Treg	Foxp3, STAT5	IL-10, TGFβ
IL-4, TGFβ	Th_9	Gata3, STAT6	IL-9
IL-6, IL-21, IL-12	T_{fh}	Bcl6, STAT3	IL-21 and IFNγ
IL-12	Tc_1	BLIMP1, Runx3	TNFα, IFNγ, granzyme, perforin
IL-4	Tc_2	Gata3	IL-4, IL-5, IL-13

Table 1.1: T cell differentiation and their signature transcription factors and cytokines.

INFγ is produced from many cells including NK cells, $CD4^+$ and $CD8^+$ T cells. Immunity to intracellular infection mostly relies on the Th_1 cells secreting IFNγ. The IFNγ mediated bactericidal activities are brought by the following mechanisms

1. Activates IDO, depleting tryptophan in the cells
2. Induces NOXs2, iNOS, and dual oxidases to synthesise reactive nitrogen species and reactive oxygen species respectively
3. Induces GTPase which helps in bacterial degradation
4. Induces MHC expression for better antigen presentation
5. Induces CD40 expression for T cell activation

Apart from the above functions, IFNγ plays a major role in $CD8^+$ T cell activation and B cell antibody production. IFNγ also induces IL-12 and TNFα production in the macrophages. The functional skewing of macrophages brought by IFNγ are termed as M_1 macrophages. **TGFβ** activates SNAIL which induces SMAD2/3, PI3K and GSK-3β pathways in the macrophages inhibiting pro-inflammatory cytokines and inducing anti-inflammatory cytokine secretion[9]. TGFβ also inhibits TCR-mediated activation of T cells. It can specifically inhibit Th_1 and Th_2 differentiation. They also control the

tolerance of the T cells[10]. It induces PD1 and CTLA4 expression in the epithelial cells and T cells thus inhibiting their antigenic responsiveness. Along with IL-10, it helps in the differentiation of Treg cells continuing the immunosuppressive cycle[6]. **IL-10** induces STAT1 and STAT3 dimerisation which induces transcription activity of anti-inflammatory genes. In macrophages counteracts the inflammatory response by down regulating MHC expression, decreasing CD40 and CD28 expression, and inhibiting the secretion of IL-12, TNFα, IL-1β, IL- and IL-8. In T cells it enhances the generation of Tregs and induces the production of TGFβ. It has a crucial role in memory T cell generation[6,11,12].

1.1 Review of literature

It been estimated that 20 out of 1000 women with chlamydial infection develop pelvic inflammatory disorder, suffer pelvic pain and 3 show infertility. 50-70 of women remain asymptomatic throughout the infection. Both symptomatic and asymptomatic have a risk of developing complications leading to infertility and PID[13]. Women suffering from PID are 7-10 fold more likely to develop ectopic pregnancy[14]. Most of these complications are the result of inflammation. It is persistent infection and ineffective immune response that contributes to the complication. Further, failure of immune activation results in the formation of aberrant chlamydial inclusion which might result in latent infection. It is thought that chlamydial HSP60 displays homology to human HSP60. Thus, the *chlamydia* HSP60 antigenic component might be activating human HSP60 cross-reactive Tregs that produce IL-10. These Tregs upon stimulation promote a switch from Th_1 to Th_2 response dampening IFNγ secretion thus delaying the resolution of infection[15].

1.1.0.1 Anti-chlamydial imunity

Chlamydia trachomatis is often recognized by TLR4 which is poorly expressed in the lower tract of the female genital tract. Chlamydial LPS and heat shock proteins are recognized by TLR4 or TLR2. However, TLR4$^{-/-}$ and TLR2$^{-/-}$ mice were able to produce cytokines on chlamydial infection indicating other strategies of recognition. The precise role of TLRs in host resistance or immunopathology is yet to be dissected. Apart from TLRs, NOD1 and NOD2 proteins can also detect *chlamydia*. These receptors are important in detecting intracellular pathogens. Previously, NOD1 is reported to *chlamydia* causing upregulation of NF-κB expression in fibroblasts. An adaptive immune response that clears the infection from the genital tract develops over a period of several weeks. Thus this leaves a gap for the re-infection or persistent infection. It is

well established that, $CD4^+$ T cells generated during primary infection provide protective immunity against chlamydia. Using mice models that lack $CD4^+$ T cells or $CD8^+$ T cells, it is shown that $CD4^+$ T cells play a major role in chlamydial clearance compared to $CD8^+$ T cells[16]. Even though both $CD4^+$ and $CD8^+$ T cells are generated in response to genital tract *Chlamydia* infection, $CD4^+$ T cell-mediated immunity is superior to $CD8^+$ mediated CTL activity. Extended investigations using KO mice have revealed the role of IFNγ, MHC II or IL-12 in protective immunity and their importance in Th_1-mediated immunity against chlamydia. Th_1-dominated immunity is protective and Th_2-mediated immunity delays the clearance of *chlamydia*. When chlamydia-specific Th_2 clones were injected into nude mice, *chlamydia* clearance was delayed[17,18]. Upon acute infection with *C. trachomatis*, CD28 required for T cell priming is upregulated and efficient Th_1 effector cells are developed for bacterial clearance. However, the Th_1 cells are reported to be responsible for the immunopathology observed during infection. Thus, it appears Th_1 development is important along with counter Th_2 immunity via IL-10 production and Foxp3+ Tregs are necessary.

IFNγ produced from $CD4^+$ T cells, $CD8^+$ T cells, and natural killer cells provide anti-chlamydial action in the genital tract. IFN$\gamma^{-/-}$ mice were unable to clear the pathogen. Very high doses observed during 1^{st} week of the infection was shown to confer protection by blocking the chlamydial growth[19]. However, low doses of IFNγ rather promoted persistent infection by promoting the aberrant RBs[20][21]. Thus, the exact mechanism and requirement of IFNγ for complete clearance of chlamydia is still incompletely understood. Major anti-chlamydial changes brought by the IFNγ are through iNOS, IDO, deprivation of Fe^{+2} via downregulation of transferrin receptors, stimulation of phagolysosome fusion, and disruption of vesicular nutrient transport[22]. Even though the strong role of IFNγ in the immunopathogenesis in chlamydial infection is well established, its production from $CD4^+$ T cells and their regulation is poorly understood. In the current work, the mechanism of regulation of $CD4^+$ T cell differentiation with respect to the involvement of macrophages is explored.

Above reports indicate that IFNγ is essential for pathogen clearance. However, the clinical cases of the chronic infection show poor Th_1 cells and Th_1 polarising cytokine IL-12. The study is taken up to explore the mechanism of the defective Th_1 generation and the consequence of these defective Th_1 cells on innate immune cells and pathogen clearance.

Hypothesis

Chlamydia trachomatis has the ability to modulate crucial host cellular pathways like cell cycle regulation and apoptosis for its survival. It also manipulates inflammatory

mechanisms of innate immune cells disturbing the adaptive immunity for its persistent growth, depleting host defense mechanisms. Residing in the macrophages, it may manipulate macrophages in such a way, it inhibits adaptive immunity and enhances anti-inflammatory phenotype in macrophages. These abilities of *C. trachomatis* drive us to hypothesize that, *Chlamydia trachomatis* could be an inducing agent in cervical cancer. We hypothesize that chronic infection-mediated immune suppression and its ability to modulate in epithelial cells are key evidence for its ability to cause cancer in the presence or absence of HPV. The study also explores the possibility of the cumulative effect of oncogenes and immunosuppressive environment brought by HPV and *C. trachomatis* leading to cervical cancer.

The hypothesis was tested by studying by analysing *C. trachomatis* mediated immune suppression and chronic inflammation (Thesis: Chapter 1) and *C. trachomatis* induced alteration in the epithelial cells in the presence and absence of HPV E6-E7 (Thesis: Chapter 2). By co-relating the chronic infection/inflammation and cellular alterations, we could provide evidence that *C. trachomatis* has a role in cervical cancer.

1.2 Materials and Methods

1.2.1 Mouse model of genital *C. trachomatis* infection

Age-matched Female BALB/c and C57BL/6 mice (6-8 wk old) were housed under specific pathogen-free conditions at the Animal house, Nirma university and Prerana Bio-Innovation Solutions. All experiments were conducted in accordance with the guidelines of the Institutional Animal Ethical Committee after obtaining the clearance. Housing and handling of mice were in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Mice were infected with 10^6 IFU (inclusion forming units) intravaginally in 100μl of sucrose phosphate buffer (SPG) buffer repeatedly as shown in the figure for chronic (C) and chronic resolved (CR) infection for 20 days. The chronic infection group of mice was sacrificed on day 20 and CR mice were sacrificed on day 60. In a replicate group of chronic mice (C), secondary infection (SI) of *C. trachomatis* was done on day 60 and sacrificed on day 65. Acute infection (A) of *C. trachomatis* was done by infecting mice once and sacrificing on day 7. In a replicate group of A, mice were housed till day 20 for infection to resolve and termed as the acute resolved group (AR). Control mice (UI) were treated with SPG buffer intravaginally. Each group consisted of 6 mice (n=6) and the experiment was repeated thrice. Bacterial shedding and infection were monitored for the presence of bacteria in cervical swabs. The bacterial burden on the day of sacrifice was analyzed in tissue and vaginal lavage (collected 1ml PBS). Age-matched

Female BALB/c and C57BL/6 mice (6-8 wk old) were infected similarly to the above-mentioned chronic group and sacrificed on day 20. Bone marrow, spleen, lymph node, blood and female genital tract were collected to analyze the macrophage polarisation and MDSC population. Serum cytokines were also measured from the collected blood.

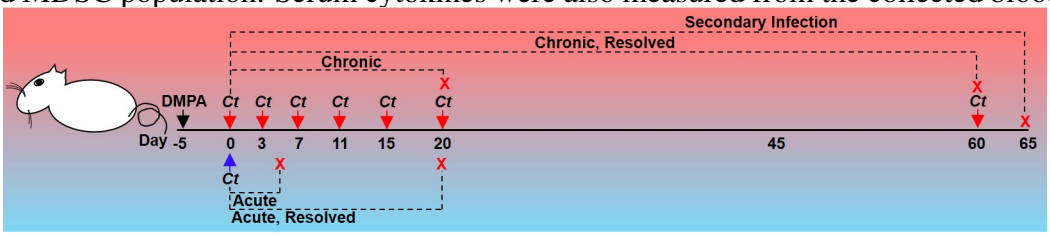


Figure 1.2: Acute, Chronic and secondary genital infections of *C. trachomatis* in BALB/c mice. Mice were injected with depo-medroxyprogesterone acetate (DMPA) intramuscularly 5 days prior to the first infection. *C. trachomatis* in SPG buffer was injected in the cervix using mosquito tips at indicated time points. Mice were housed in pathogen-free conditions till the sacrifice day.

1.2.2 Bacteria and Bacterial lysate

Chlamydia trachomatis serovar D was procured from ATCC (VR-88). *Chlamydia* was cultured as previously described[23]. Briefly, frozen vials containing *C. trachomatis* EBs in 4SP (0.4M sucrose, 16 mM Na_2HPO_4) were thawed and inoculated to overnight grown confluent McCoy cells and observed for inclusion body formation. Cells were incubated in 5% CO_2 incubator at 37°C for 48hrs. Cells were washed with PBS and scrapped to collect the cells containing *C. trachomatis* EBs. Cells were lysed and EBs were purified from cell lysates by 20%-50% Urograffin gradient centrifugation and stored in 4SP buffer at -80°C till further use. Inclusion forming units (IFU) of every batch were monitored before the experimentation. IFU vs *Ct* DNA 16S rRNA ΔΔCt values were normalized and used to determine the MOI during infections. 10^7 *C.trachomatis* EBs were lysed in 0.1% tween at 70°C for 20min to prepare *C.trachomatis* lysate which was later used as antigen (*C.tr*-Ag).

1.2.3 *C. trachomatis* infection and maintenance

EBs are added to cells at an MOI of 1 and centrifuged at 750g for 30min. After centrifugation, the media was replaced with fresh media without antibiotics. Infected cells along with controls (4SP buffer only) were incubated for indicated time points.

1.2.4 DNA isolation and qPCR for bacterial burden

DNA was isolated from infected cells by lysing the cells in lysis buffer (10%SDS in TE buffer containing RNAse and Proteinase K). The phenol-chloroform method was

used for DNA extraction. Extracted DNA solubilised in TE buffer. DNA was used as a template for qPCR of mouse GAPDH gene and *C. trachomatis*16S rRNA gene. The ratio of 16S rRNA/mouse GAPDH was used to assess bacterial burden. The 16S *C.tr* rRNA/mouse GAPDH ratio vs inclusion forming units (IFU) vs MFI standard graph was plotted which was used later to determine bacterial burden.

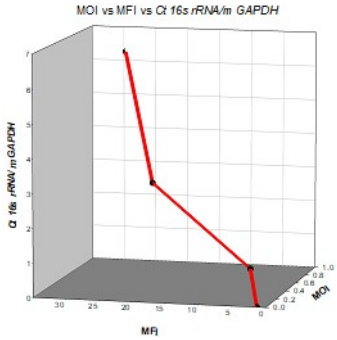


Figure 1.3: 16S *C.tr* rRNA/mouse GAPDH ratio vs inclusion forming units (IFU) vs MFI standard graph. Mouse fibroblast cells were infected with *C. trachomatis* at various doses and analysed for bacterial burden using *Chlamydia* OMP immunofluorescence and genomic content. MFI was calculated from confocal images and genomic content by qPCR analysis of Ct 16s rRNA gene content from DNA. The graph was plotted to establish co-relation (n=3)

1.2.5 Other Techniques used in the study

The routinely used techniques are not explained in detail. However, the procedures have been done from well-established protocols which are explained in detail in the thesis.

Flow cytometry	T cell characterization, Macrophage polarisation and MDSCs[24]
Membrane purification	Ultracentrifuge[25]
Raft and Non–raft	Ultracentrifuge[25]
Lipid analysis	HPLC[26]
Co–localisation	Confocal microscopy
Protein levels of cell signaling molecules	Western Blotting
mRNA levels	sqRT–PCR and qPCR
Protein levels in tissue	Immunohistochemistry
Apoptosis	Annexin V–FITC/PI
Cell proliferation	MTT assay, BrdU incorporation and Ki67 expression
Cell cycle analysis	PI Staining (Flow cytometry)

1.3 Results and Disucssion

1.3.1 Live *C. trachomatis* induces M_2 macrophages

Previously, *C. trachomatis* infected patinets show increased IL-10 levels in the serum, However, their source or implications has not be explored. To understand the role of *C. trachomatis* mediated manipulation of host cells on immune cells, macrophages were infected with live *C. trachomatis* and compared with equal antigenic components derived from heat killing *C. trachomatis*. The antigenic component of *C. trachomatis* induced inflammation, while the live bacteria induces anti-inflammatory cytokines. Only the *C. tr*-antigen primed macrophages were able to functionally skew to M_1 macrophages(MHC-II^{high} which was accompanied with increased TNFα and IL-12 levels. Where as the live bacteria prevents inflammatory output in the macrophages and indeed polarises them to M_2 macrophages (CD206^{high}). The functinal skewing is also results in *C. trachomatis* infected macrophages increased Arginase-1 levels decreasing NO levels needed for bacterial clearance.

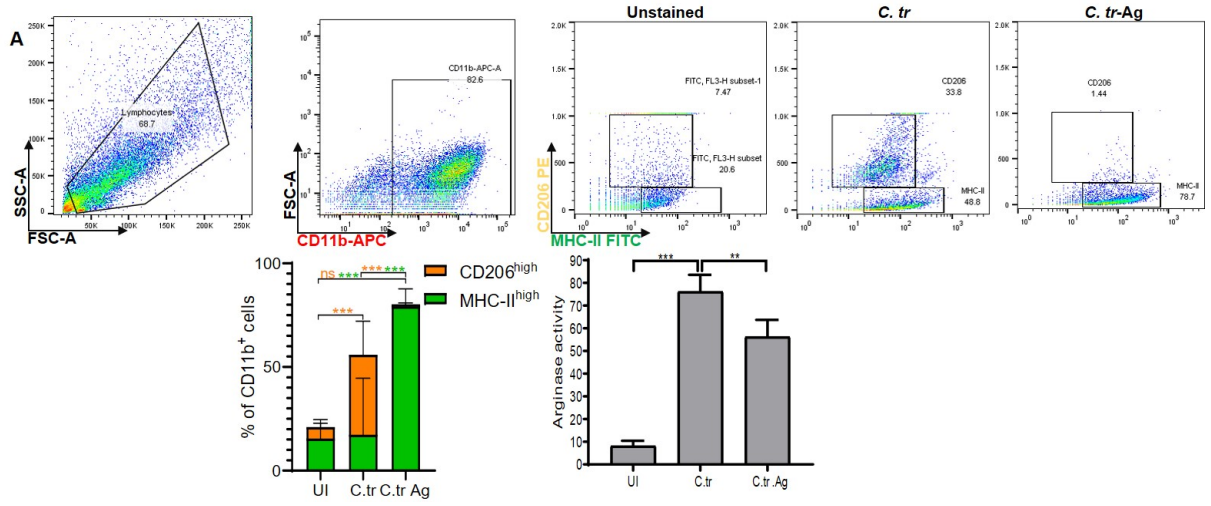


Figure 1.4: Live *C. trachomatis* induces M_2 polarisation in macrophages. Bone marrow-derived macrophages were either infected with *C. trachomatis* at MOI of 1 or primed with an equal amount of heat-killed *C. trachomatis* and incubated for 24hrs. (A) Cells were stained for surface expression of CD11b, CD206, and MHC-II. Percentage of CD11b$^+$ MHC-II$^+$ and CD11b$^+$ CD206$^+$ cells were plotted as M_1 and M_2 respectively. (B) From a parallel experiment, cells were collected and arginase activity was measured from total cell lysate and plotted as urea formed μM/min/μg of protein. (n=3; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

Futher, M_2 macrophages show the highest bacterial burden compared to M_0 and M_1 infected macrophages. Inflammatory macrophages, M_1 were able to control the growth of *C. trachomatis* as shown with decreased bacterial burden in M_1 macrophages. Upon analysis of mRNA levels, it is observed that expresses high *Ephrin B2* and *PDI* levels. These proteins were previously reported to be involved in pathogen internalization in-

cluding *C. trachomatis*, *Leishmania*, *Shigella* e.tc,[27,28,29,30,31,32] . Thus, the increased presence of these receptors makes M_2 macrophages available for bacterial internalization and thus increased burden. Further, as these are anti-inflammatory macrophages, they express low NO levels contributing to poor bactericidal effects[33,34].

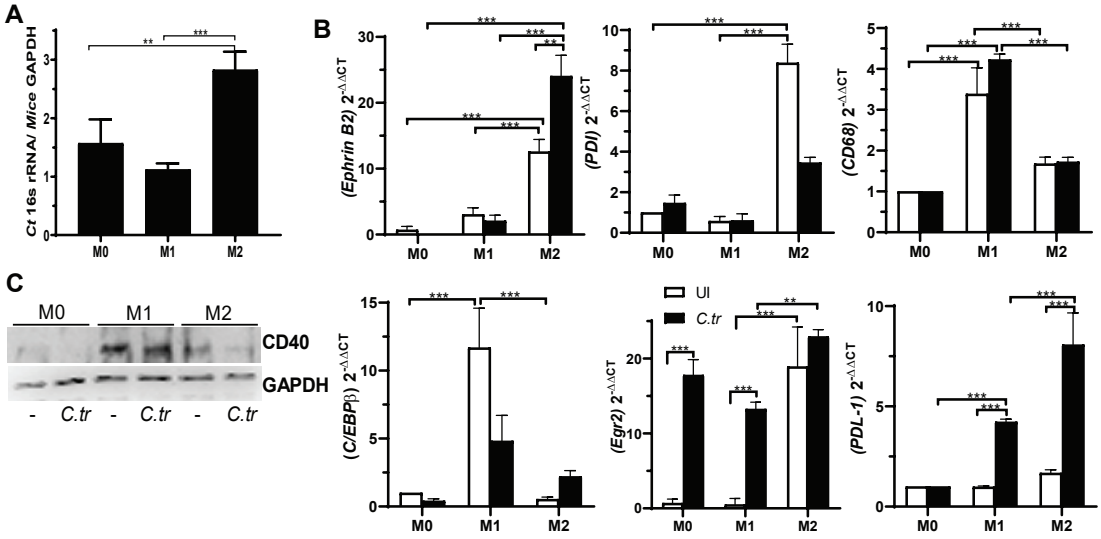


Figure 1.5: *C. trachomatis* deferentially infects macrophage subsets. Low-density bone marrow progenitor cells were cultured in presence of M-CSF to derive naïve macrophages. M_1 and M_2 macrophages were generated using GM-CSF and IFNγ; and M-CSF, IL-10, and IL-4 respectively. The polarised macrophages were infected with *C. trachomatis* and 36hrs later bacterial burden was analysed by quantifying genomic DNA of *C. trachomatis* and mouse GAPDH using qRT-PCR. Bacterial burden was analysed as the ratio of the genomic DNA (A). In a parallel experiment, 8hrs p.i cells were collected in triZOL and mRNA levels of indicated genes were analysed using qRT-PCR (B). 10^6 BMDMs were plated in 6 well plate and polarised to M_1 and M_2 macrophages as mentioned above. 18hrs p.i with MOI 1 of *C. trachomatis*, CD40 expression was analysed using Western blotting (C). (n=3; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

1.3.2 CD40 activation during *C. trachomatis* infection promote anti-inflammatory phenotype

CD40 acts as a co-stimulatory molecule activating CD40L, which is necessary for T-cell activation. It also gets activated on interaction with CD40L which initiates a chain of signaling events leading to the activation of MAPkinase pathways thus participates in fine control of IL-10 and IL-12 secretion[35].

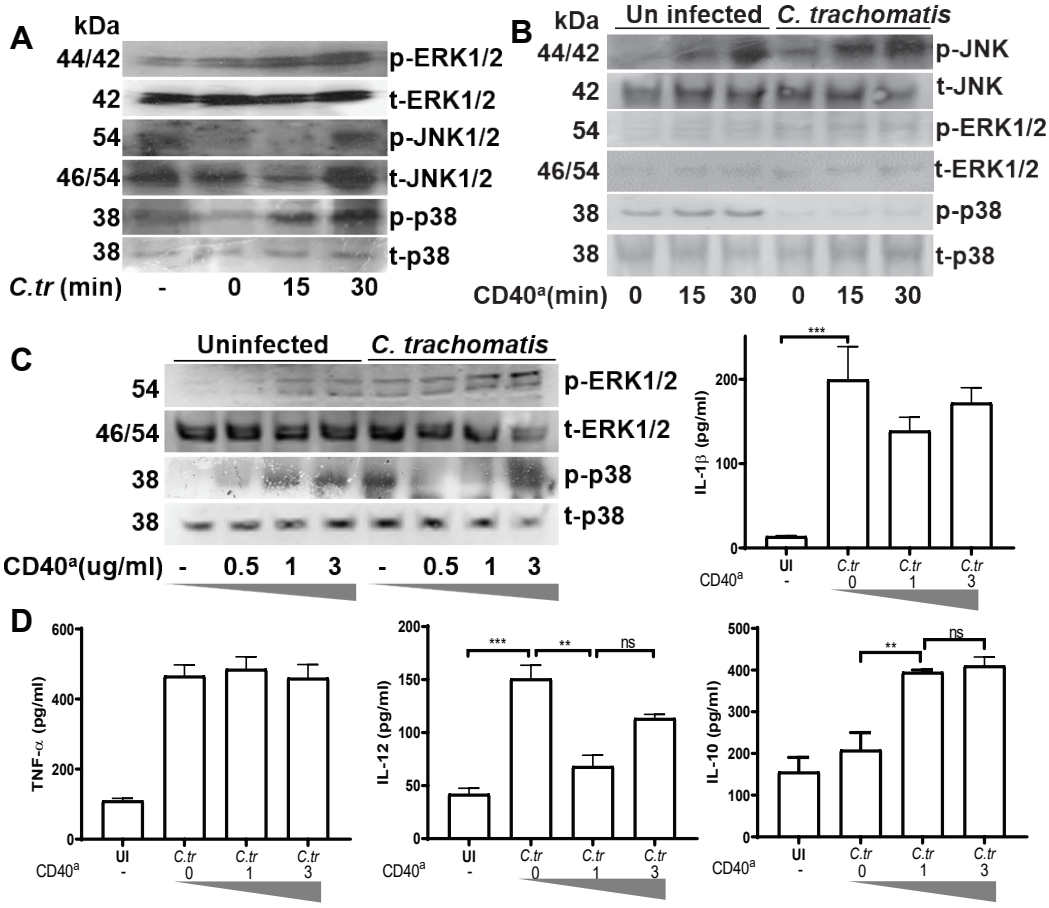


Figure 1.6: CD40 activation during *C. trachomatis* infection enhance IL-10 secretion via ERK1/2 pathway. Bone marrow-derived macrophages were infected with *C. trachomatis* and incubated for indicated time points. Total and phosphorylated forms of proteins were analyzed by western blotting (A). *C. trachomatis* infected and control cells were treated with 3μg/ml of CD40 activating antibody (CD40^a) and analyzed for total and phosphorylated forms of JNK, ERK and p38 proteins in time-dependent way (B) and dose-dependent way(C). Further, control and infected cells were treated with various doses of CD40 activating antibody (CD40^a) and analyzed for secreted cytokines from the supernatant by ELISA (D). (n=3; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

As the *C. trachomatis* infected macrophages express less CD40 compared to antigen primed macrophages and the skewing of macrophages to M_2 phenotype which are known to have low CD40 levels, we studied the consequence of the manipulation of CD40 by *C. trachomatis*. The consequences could be on both the macrophages and the T cells as CD40-CD40L is bidirectional signal. The CD40 activation which is poorly expressed lower on the macrophages during *C. trachomatis* infection resulted in an increase in ERK1/2 phosphorylation in a time- and dose-dependent manner. Simultaneously, p38 phosphorylation was decreased on CD40 activation during *C. trachomatis* infection. Consequent to ERK1/2 pathway activation, IL-10 secretion was increased on CD40 activation. IL-12 levels were decreased on CD40 activation in *C. trachomatis* in-

fected macrophages. These results indicate that, *C. trachomatis* infection induce IL-10 secretion and this effect is further enhanced by CD40 activation (Fig.1.6).

It was previously well established that *C. trachomatis* sequesters host sphingolipids for its survival. However, the consequence of the sequestration was not studied. We analysed the effect of *C. trachomatis* mediated lipid manipulation on CD40 signaling. *C. trachomatis* infection alters the plasma membrane fluidity as observed by an increase in $\nu_{sym}CH_2$ at 2850 cm^{-1}. The change in this fluidity affects the receptor signaling at the membrane by raft or non-raft formation. Alteration of ceramide and cholesterol in the plasma membrane by *C. trachomatis* facilitates CD40 mediated IL-10 secretion. It was further confirmed by microscopic observation of cholesterol and ceramide staining in uninfected and infected macrophages. Ceramide supplementation enriches the inclusion membrane with ceramide but not the plasma membrane suggesting a strong sequestration capabilities of the . Activation of CD40 in such conditions facilitates non-raft formation. Cholesterol supplementation preferentially gets enriched at the plasma membrane. Upon CD40 activation, CD40 and cholesterol co-localize, while βMCD and cycloserine disrupt this via decreasing the cholesterol and ceramide levels in the plasma membrane. All these changes induce ERK1/2 phosphorylation-mediated IL-10 secretion as shown earlier. Inhibition of ERK1/2 abrogated the IL-10 secretion on CD40 activation, indicating CD40 mediated ERK1/2 is solely responsible for the IL-10 secretion on CD40 activation during *C. trachomatis* infection.

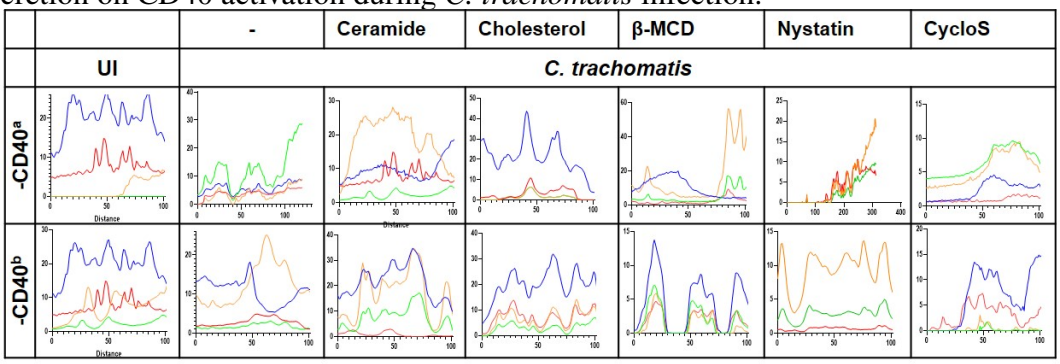


Figure 1.7: CD40 translocates to GM1 rich domains during *C. trachomatis* infection. Cells were either pre-treated with indicated lipids or synthetic inhibitors. 2hrs p.i, cells were treated with CD40 activating antibody ($CD40^a$) and incubated for 15 more min. Cells were washed with PBS and labeled using anti-CD40 conjugated with PE, anti-CD11b conjugated with APC. The presence of GM1 was detected using FITC-conjugated cholera toxin. Cholesterol was stained with filipin. Cells were fixed and observed under Leica confocal microscopy under 60X magnification. A minimum of 10 sections were taken in Z-axis. Randomly selected images were presented in the figure. The expression of individual protein or lipids were anlaysed and plotted as a graph. Co-localized proteins/lipids show similar patterns or overlaps in the graph indicating their presence in similar regions. (n=3)

1.3.3 Chronic *C.trachomatis* induces M_2 macrophages

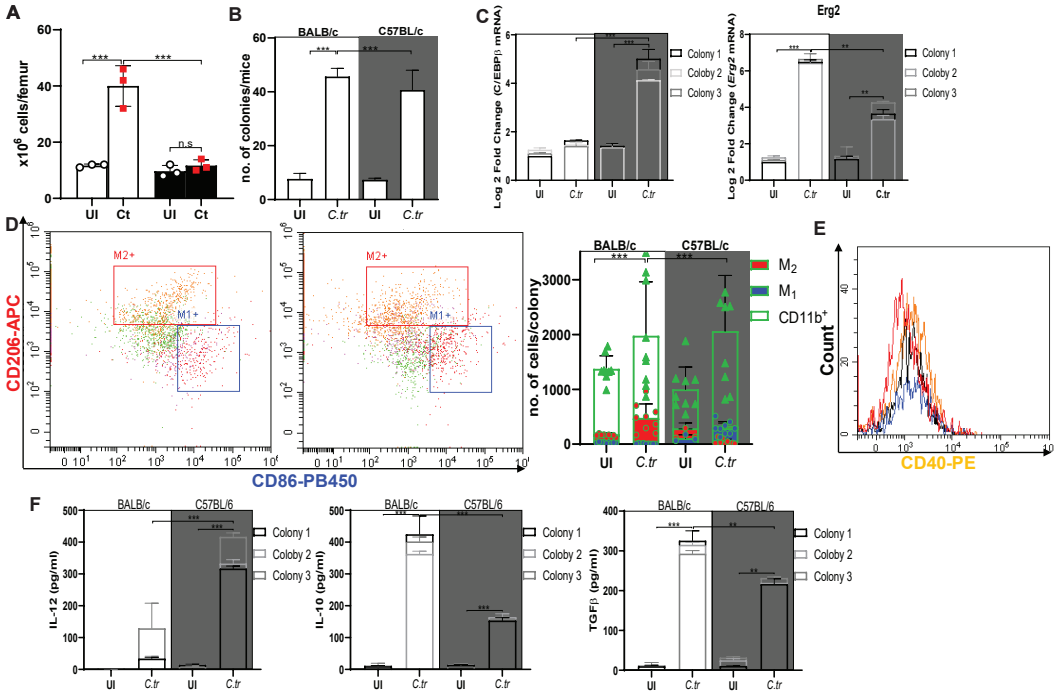


Figure 1.8: BALB/c mice show increased myelopoiesis during *C. trachomatis* which differentiate to M_2 macrophages. Bone marrow cells were isolated from the femur of the control and infected BALB/c and C57BL/6 mice. No. of cells from each femur is plotted (A). An equal number of bone marrow cells were grown in Isovac's modified medium containing 5% FBS 3% methylcellulose, IL-3, and 10μg M-CSF. No. of colonies formed after 7 days of incubation was counted and plotted (B). The colonies formed in the above experiment were analysed for mRNA expression of *C/EBPβ* and *Erg2* by qRT-PCR (C). Three colonies from each group were picked and stained for CD11b, CD206, and CD86. $CD11b^+$ cells were analysed for CD86 and CD206 expression. The number of positive cells were plotted (D). These differentiated macrophages ($CD11b^+$) were analysed for CD40 expression(E). (n=3; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

To understand the consequence of chronic infection on myelopoiesis, we have isolated bone marrow (BM) cells from both BALB/c and C57BL/6 uninfected and infected mice and cultured them in presence of M-CSF and IL-3 which serves as a common proliferation signal and driver of macrophage differentiation. Chronic *C.trachomatis* infection increased the number of bone marrow cells in the infected mice and further show increased colony-forming units compared to the uninfected mice indicating their commitment to proliferation and differentiation. The myelopoetic colonies formed in each group were analyzed for mRNA levels of *C/EBPβ* and *Erg2*. These serve as transcription factors controlling the inflammatory and anti-inflammatory genes in M_1 and M_2 macrophages respectively. Colonies from the *C.trachomatis* infected C57BL/6 mice showed increased levels of *C/EBPβ* mRNA levels indicating its commitment to M_1 macrophages. Myelopoetic colonies from bone marrow obtained from *C.trachomatis* infected BALB/c mice show higher *Erg2* levels indicating their M_2-like

properties. Previously, bone marrow-derived macrophages from BALB/c on infection with *C.trachomatis* also showed increased Erg2 levels[36]. These colonies were further individually cultured in a 96 well plate to analyze their secretome. BM cells from infected BALB/c mice secreted high levels of IL-10 and TGFβ. While BM cells from infected C57BL/6 mice secreted high levels of IL-12. The cytokine profile from the cells correlates with the *C/EBP*β and *Erg2* mRNA levels.

We have analyzed the presence of MDSC cells in the bone marrow by flow cytometer. MDSCs expressing Ly6C and Ly6G were increased in the bones of BALB/c mice on infection with *C. trachomatis*. It is observed that *C. trachomatis* infection specifically increased M-MDSC ($CD11b^+$ $Ly6C^+$ $Ly6G^+$) in BALB/c mice. Even though infected C57BL/6 mice also showed an increase in MDSC population at the bone, their percentages are lower compared to infected BALB/c mice and their phenotype is of G-MDSC ($CD11b^+$ $Ly6C^{++}$ $Ly6G^+$) phenotype. We have characterised the splenic and circulating MDSCs also which are shown to be increased in the BALB/c mice on infection with *C. trachomatis*. These increased MDSCs can contribute to the immune suppression and poor bacterial clearance observed in the BALB/c mice. They can also differentiate to M_2 like macrophages at any stage. This could be one of the reasons for an increased presence of M_2 macrophages in the spleen and cervix of BALB/c mice during *C. trachomatis* infection.

1.3.4 Acute and chronic genital *C.trachomatis* infection show differential immune response

These chronic and secondary infections were compared to acute and acute resolved infections. The acute infection demonstrates high *C.trachomatis* as observed by its presence in the swab, lavage, and tissue. The acute resolved (AR) infection mice showed *C.trachomatis* persistence as seen observed by the presence of bacteria in the swab, lavage, and cervical tissue. These results indicate that chronic infection showed poor clearance of *C.trachomatis* indicating poor elicitation of recall response (Fig.**??**B-D). Repeated infection groups (C, CR and SI) not only showed poor bacterial clearance but also show fluid accumulation and tissue destruction as an indicator of inflammation (Fig.1.10A). The infected mice showed increased spleen and lymph node volumes and weights compared to naïve mice indicating immune elicitation.

IFNγ plays a major role in immune activation and bacterial clearance. This with other cytokines gets released into the blood from various cells which acts on various cells at distant sites to exert their actions. The measure of cytokine in the blood/serum indicates the immune activation by the whole organism. The serum IFNγ levels were monitored during the infection. Serum IFNγ levels peaked at day 7 with closer to 3ng/ml

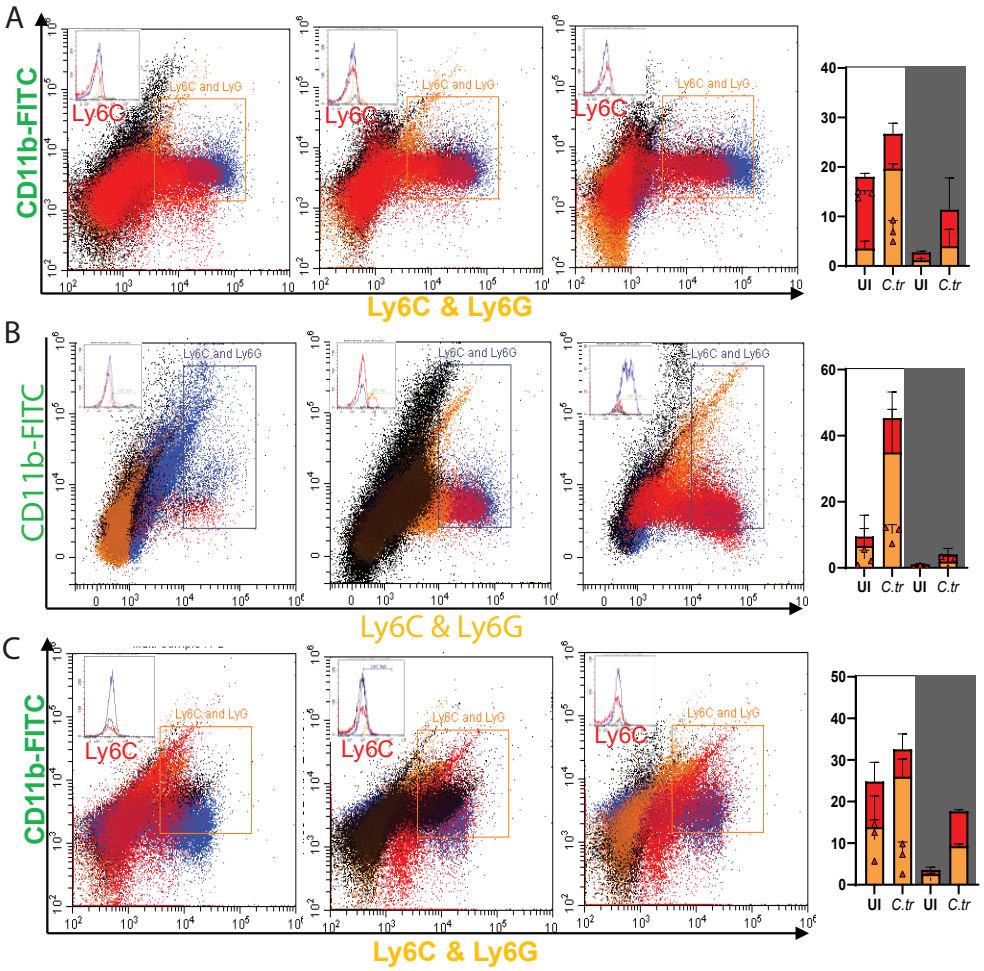


Figure 1.9: *C. trachomatis* infection induces MDSC in BALB/c mice. Bone marrow cells from chronic *C. trachomatis* infected BALB/c and C57BL/6 were isolated and stained for CD11b and Ly6C&Ly6G with a common antibody labeled with PE. Ly6C were separated and stained with Ly6C specific antibody labeled with APC. Ly6C levels on MDSC were analysed to differentiate M-MDSC & G-MDSC (A). From same mice, blood (B) and spleen (C) were analysed for M-MDSC & G-MDSC population.(n=3; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

which reduced with time in acute infections. In chronic infection mice, the levels were lower ranging in 1-2ng/ml compared to acute infection (3ng/ml). On secondary infection, IFNγ in the serum were negligible compared to other groups and correlates with poor pathogen clearance. The humoral immune response is mediated by antibodies, which are higher in mice with acute *C. trachomatis* infection. Serum IgG levels were higher during acute and chronic infection which decreased during the resolution phases (A and AR). On secondary infection, IgG levels were increased in serum, but the levels were lower compared to acute and chronic infection. Secretory antibodies in the secretions play an important role in mucosal immunity to fight the invading pathogens[37]. Likewise, IgA levels were increased in the acute infection which reduced with time

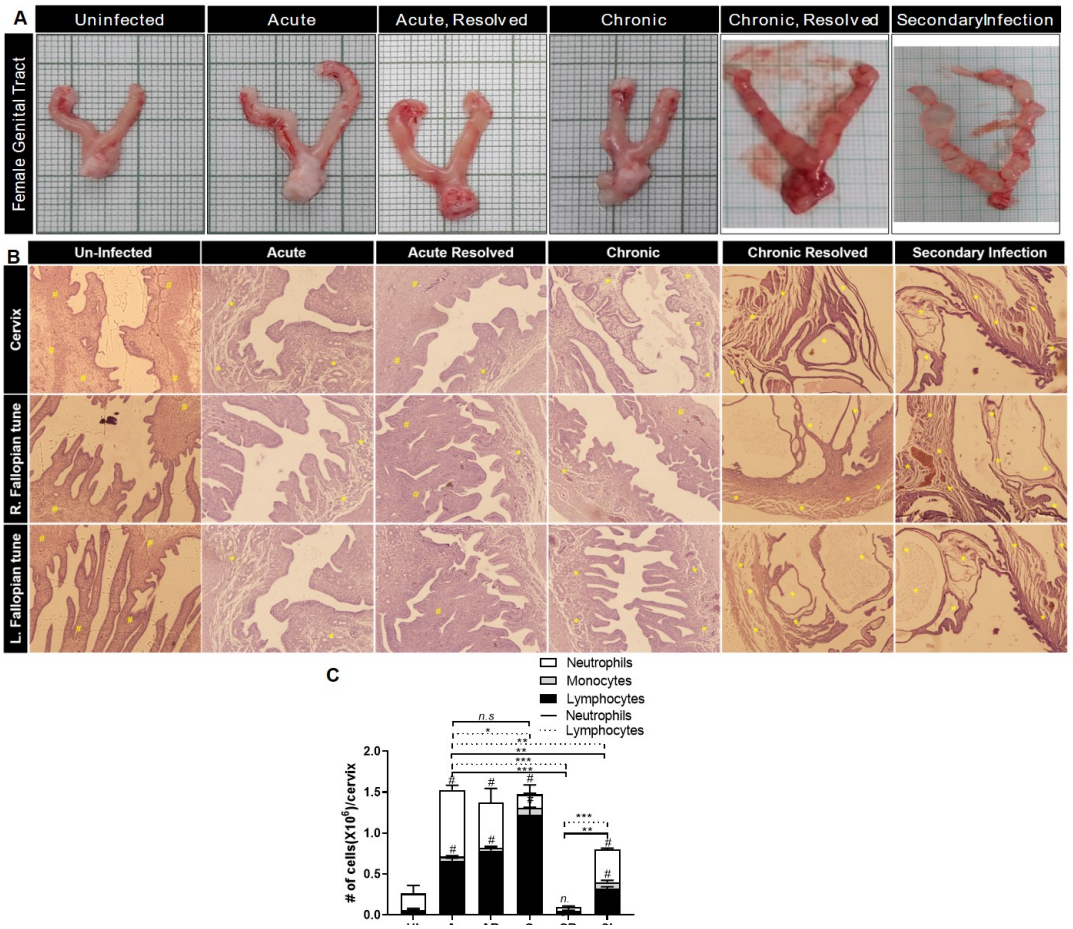


Figure 1.10: Tissue pathology and immune cell infiltration in acute and chronic mouse models of *C. trachomatis* geital infection. Balb/c mice were genitaly infected with *C. trachomatis* as mentioned in M&M. The female genital tract was collected and analysed for its pathological appearance (A). H&E staining of the female genital tract was done(B). Vaginal lavage was analysed for differential WBC count and plotted as a number of cells collected from the cervix (C).(n=6; ***indicates ρ<0.0001; **indicates ρ<0.005; **indicates ρ<0.01).(n=6; ***indicates ρ<0.0001; **indicates ρ<0.005; **indicates ρ<0.01).

(AR). On chronic infection, other cell-mediated immune responses take over due to prolonged infections. IgA levels were reduced during chronic infection. IgA which plays an important role in pathogen clearance is not increased during secondary infection indicating a crooked immune activation. Even the inflammatory cytokines, TNFα, and IFNγ were not increased during secondary infection. During acute infections, TNFα and IFNγ which help in pathogen clearance are increased along with IL-10. These cytokines were maintained in higher concentration for 20 days helping pathogen clearance. These cytokines were taken over by the IL-10 and TGFβ during chronic infection. During secondary infection, all the cytokines concentrations were lower compared to acute infection indicating poor immune elicitation. Failure in the IFNγ and other inflammatory cytokine secretions which are necessary for bacterial clearance resulted in poor

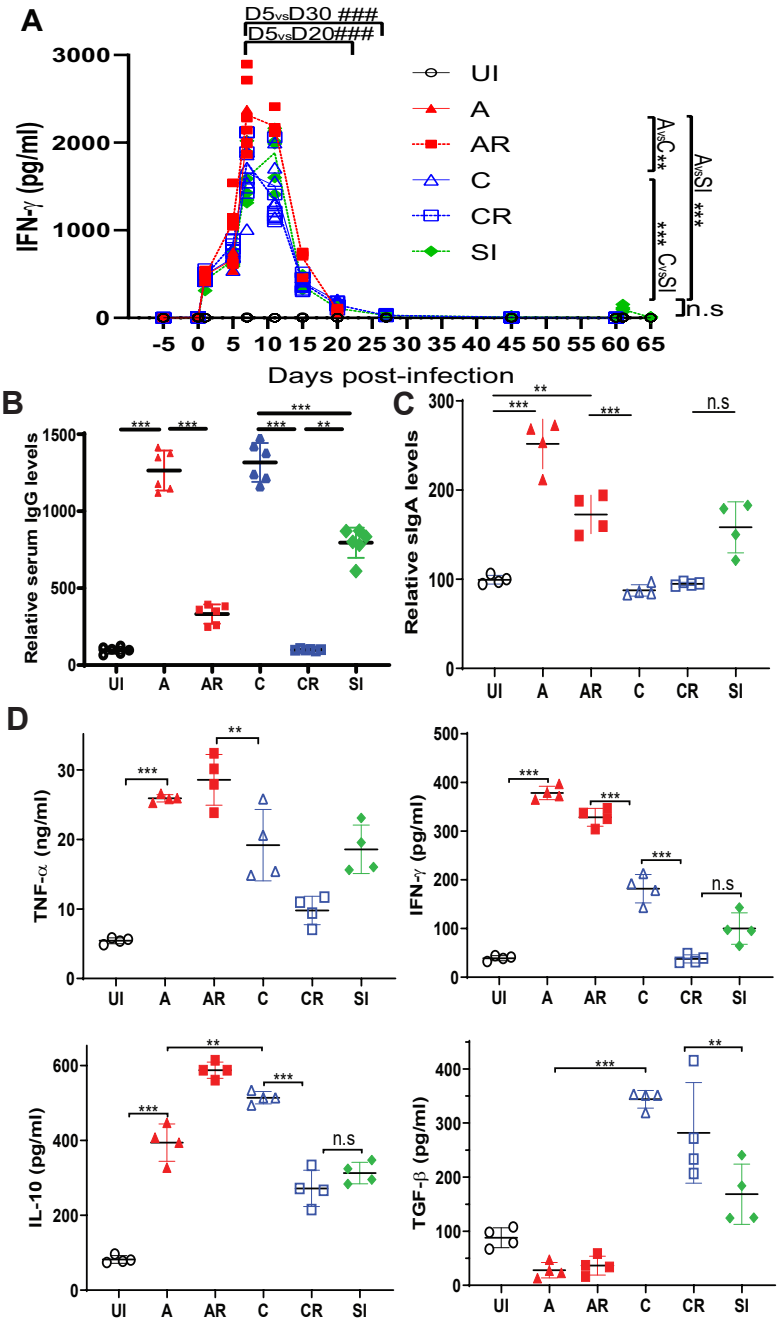


Figure 1.11: IFNγ insufficiency during chronic and secondary *C. trachomatis* infection. Serum was collected every 4 days from retro-orbitally collected blood and analysed for IFNγ (A). IgG levels were measured in the serum and plotted as relative levels (B). IgA levels were measured from vaginal lavage and plotted as relative levels (C). Cytokines: TNFα, IFNγ, IL-10 and TGFβ were measured from the vaginal lavage collected from infected and control mice. (n=6; ***indicates ρ<0.0001;**indicates ρ<0.005; **indicates ρ<0.01; n.s=non significant)

bacterial clearance as observed in Fig.**??**. Simultaneously the increase in IL-10 and TGFβ during chronic and secondary infections could be contributing to the establishment of immunosuppression-mediated infection.

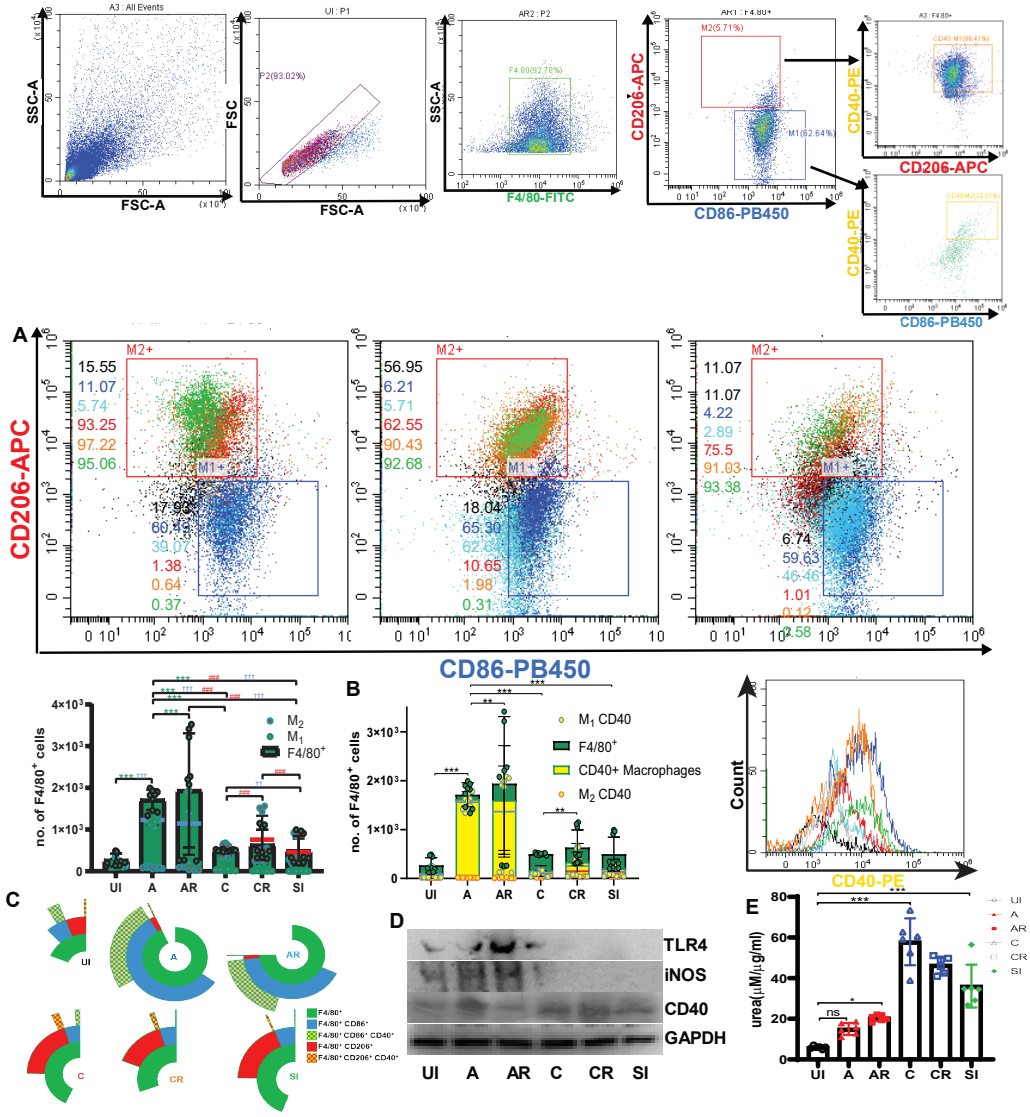


Figure 1.12: M_2 macrophage are recruited at female genital tract during chronic *C. trachomatis* infection. The genital tract of infected and control mice was weighted and minced in PBS. Cells collected from each mouse were stained for macrophage markers. Cells collected from the minced tissue were re-suspended in constant volume and all the cells were acquired. F4/80, CD86, and CD206 expressions were analyzed using respective antibodies labeled with FITC, PB450, and APC respectively. $F4/80^+$ cells were considered as macrophages; $F4/80^+$ $CD86^+$ were considered as M_1 macrophages and $F4/80^+$ $CD206^+$ were considered as M_2 macrophages. The total number of cells positive for respective markers were plotted (Symbols over the line represent ρ value significance, * for $F4/80^+$; for M1 and † for M2) (A). CD40 expression on the recruited macrophages was analyzed by flow cytometry and plotted as $CD40^+F4/80^+$ cells. Additionally, CD40 expression on M1 and M2 macrophages were plotted to enumerate the distribution. Representative histograms of CD40 expression on cervical infiltrated $F4/80^+$ cells were plotted (B). The phenotypic and CD40 distribution in the cervical infiltrated macrophages were plotted as a sunburst graph of mean values obtained from the individual group of mice (C). CD40 and iNOS expression levels were observed using western blot from the RIPA lysed for the tissue of infected and control mice (D). Simultaneously tissue was lysed in PBS used to measure arginase activity by measuring the conversion of arginine to urea per ug of protein per ml (E). (n=6; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01; n.s=non significant)

The immunosuppressive cytokines found in the vaginal fluid could be secreted by macrophages and/or T cells. To assess the source of the immunosuppressive cytokines and the role of macrophages in immune suppression, the macrophage population recruited to the female genital tract were analyzed for M_1 and M_2 markers by analyzing CD86/CD68 and CD206 expression on $CD11b^+$ macrophages respectively. *C. trachomatis* infection induces recruitment of the macrophages to the female genital tract. Their levels and phenotype vary during primary acute and chronic infections and secondary infections. During acute infections, macrophage recruitment is higher and the recruited macrophages reside at the site of infection till day 20 as observed in the acute resolved group. The macrophages recruited were of M_1 phenotype. Chronic infection show decreased macrophage infiltration and the phenotype is of M_2 phenotype. Chronic infection resolution is assisted by the M_2 macrophages which were of high number at the cervix. The secondary infection fails to induce macrophage infiltration. The lower number of macrophages that are recruited were M_2 macrophages with low CD40 expression.

The recruited macrophages dictate the fate of T cells. Total protein was isolated from the tissue which was used to analyze the arginase 1 activity as an indicator of M_2 macrophages present at the tissue. Acute infections show low arginase activity while chronic and secondary infections show high arginase activity correlating with M_1/M_2 macrophage population observed by flow cytometry. During chronic infection, as macrophages skews to M_2 phenotype, the CD40 expression also gets decreased. Even the CD40 expression in overall tissue is observed to be low compared to acute infection. This might be due to the low availability of IFNγ (a CD40 inducer) in chronic and secondary infections. The surface expression analyzed by flow cytometry correlates with the CD40 expression analyzed by western blot and IHC. Western blot analysis also showed that chronic infection expresses low iNOS levels indicating the lower M_1 macrophages abundance at the cervix.

After a period of time, the immune response is taken over by the adaptive immune cells to concentrate the activation to a pathogen-specific immune response. T cells play an important role in the pathogen-specific adaptive immune response with their helper activities by T helper (Th) cells and cytotoxic activity by cytotoxic T (Tc) cells. Once, the innate immune cells engulf the pathogen, they travel to lymph nodes where they activate T cells which leave the lymph node to reach the site of infection where they exert their action[38,39]. Depending on the cytokine milieu and stimulatory molecules the T cells recruited to the site of infection show functional divergence. To understand the role of T cells at the female genital tract, T cells were characterized based on the expression of intracellular cytokines, transcription factors (Tbet and Foxp3), and surface expression of CD4 and CD8 molecules. On the characterization of T cells ($CD3^+$) recruited at the

cervix, it is observed that T cells are mostly Th_1 secreting IFNγ in acute *C.trachomatis* infected mice. Even $CD8^+$ T cells contribute to IFNγ positivity. These cells might be the reason for the increased concentrations of IFNγ observed in the vaginal lavage from mice of the acute infection group. These cells eventually reduced over time as observed in acute resolved group. The Th_1 cells in acute resolved group were half of the number found in the acute group. The chronic infection groups also showed a lesser number of Th_1 cells compared to acute infection. These cells are replaced by the increased presence of Th_2 and Treg cells. During the resolving phase, T cells were reduced most as the infection was completely subsided or immune activation is suppressed. The secondary infection is where T cells in the lymph nodes migrate quickly to the female genital tract and act on the pathogen. The T cells recruited in this group of mice were Tregs which explains the decreased IFNγ and increased TGFβ levels in the vaginal lavage during secondary infection (Fig.**??**). These results suggest that during chronic and secondary infection of *C. trachomatis*, T cell activation is low and even the minimum activation resulted in the generation of immunosuppressive T cells. This could be due to high M_2 macrophages found at the cervix which facilitate the differentiation of Treg from naïve T cells[38,40,41].

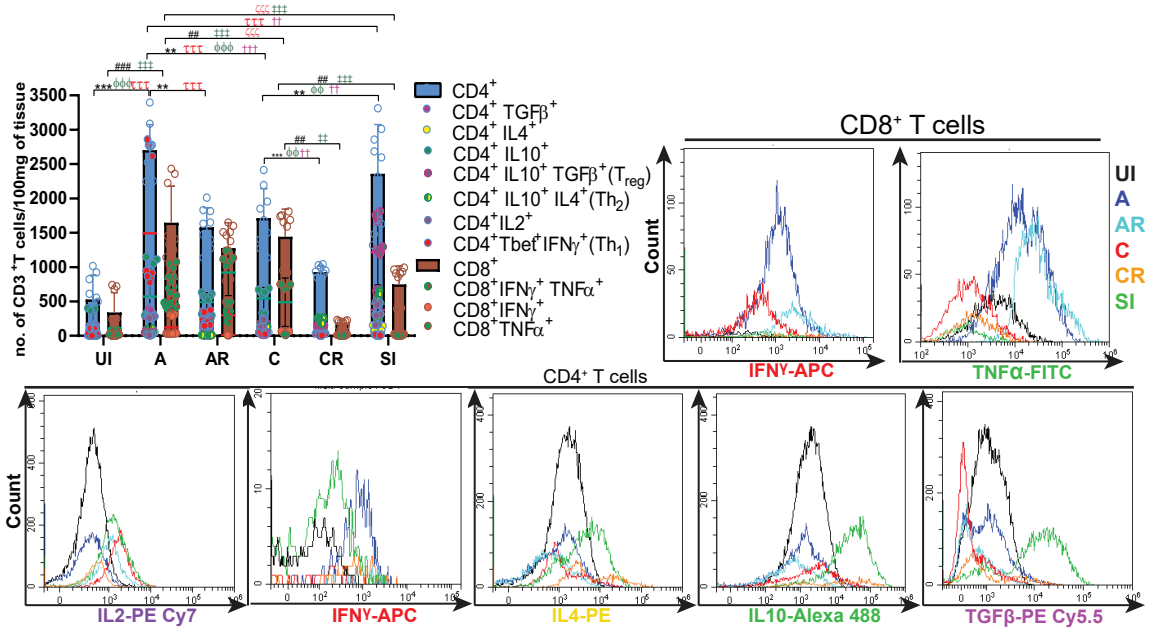


Figure 1.13: Increased presence of Treg at the female genital tract during *C. trachomatis* infection. The total number of T cells collected from each mouse was plotted(A). Histograms of cytokines from $CD4^+$ and $CD8^+$ T cells is shown(B). (n=3; M_1 vs M_1-*; M_2 vs M_2-#). The distribution of macrophage phenotype and CD40 expression in the spleen was plotted as a hierarchical sunburst graph (C). (n=6; *indicates ρ value of $CD4^+$ T, ϕ indicates ρ value of $CD4^+$ IL-10^+, τ indicates ρ value of Th_1 # indicates ρ value of $CD8^+$ T cells, ψ indicates ρ value of IFNγ^+ $CD8^+$ T cells. ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

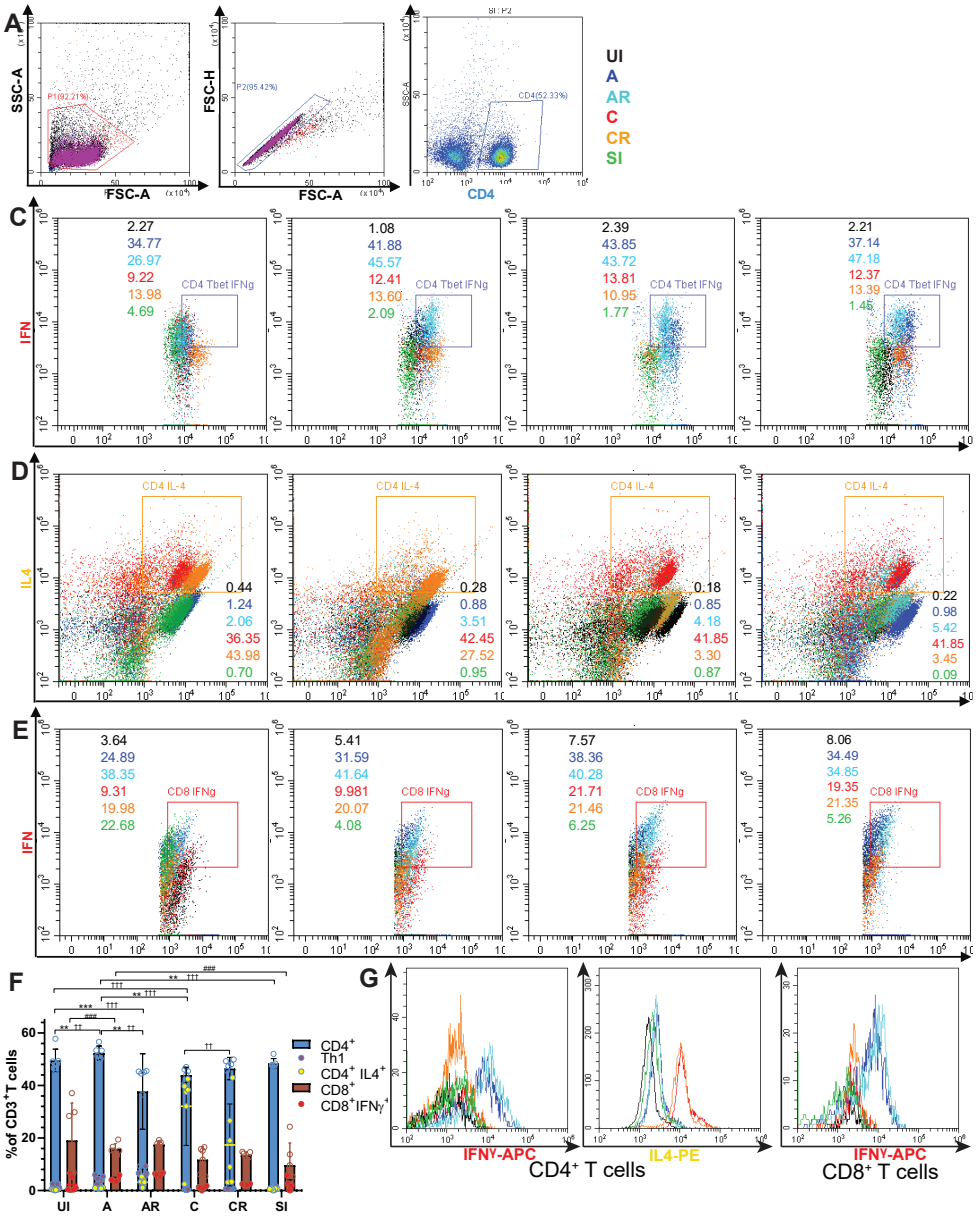


Figure 1.14: Chronic *C. trachomatis* infection reduces Th_1 cells in the inguinal lymph nodes of infected mice. Inguinal lymph nodes were collected and single-cell suspensions were prepared. Gating strategy for the T cell characterization is shown (A). $CD4^+$ $Tbet^+$ $IFN\gamma^+$-Th_1 cells were gated from inguinal lymph nodes (C). IL-4^+ secreting $CD4^+$ cells were plotted (D). $CD8^+$ T cells secreting $IFN\gamma^+$ were gated from $CD3^+$ Inguinal lymph node T cells as shown(E). % of Th_1, and IL-4^+ $CD4^+$ T cells from $CD4^+$ T cells were plotted (F). Overlay Histogram of $IFN\gamma$ expression in $CD4^+$ and $CD8^+$ T cells (G). ($CD4^+$ vs $CD4^+$-*; Th_1 vs Th_1-†; $CD8^+$ $IFN\gamma$+ vs $CD8^+$ $IFN\gamma^+$-#). (n=4; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

1.3.5 *C. trachomatis* infection alters cell cycle in epithelial cells

C. trachomatis has been reported to alter host cells for their survival in many ways. This is achieved by interfering with host cellular functions and processes including cell

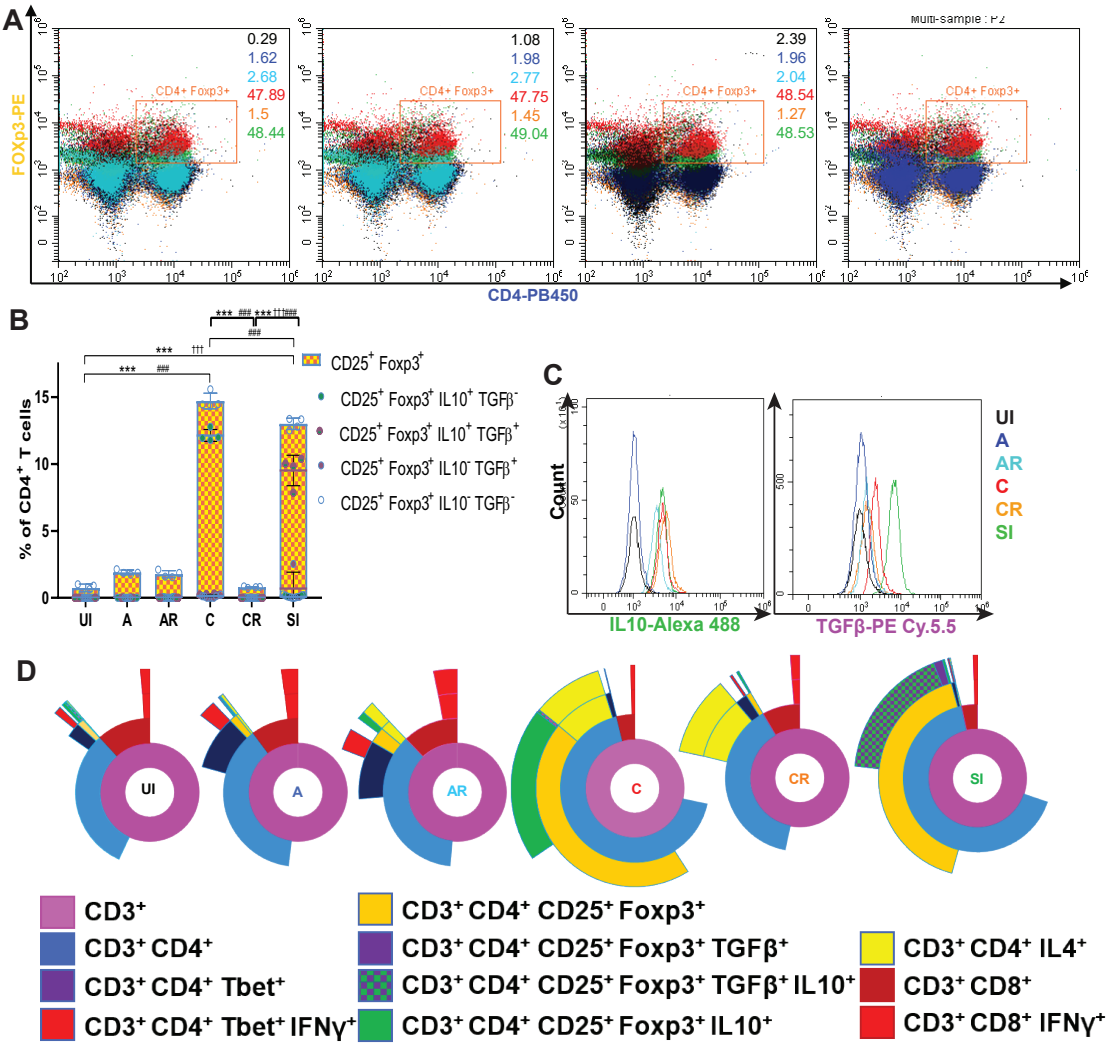
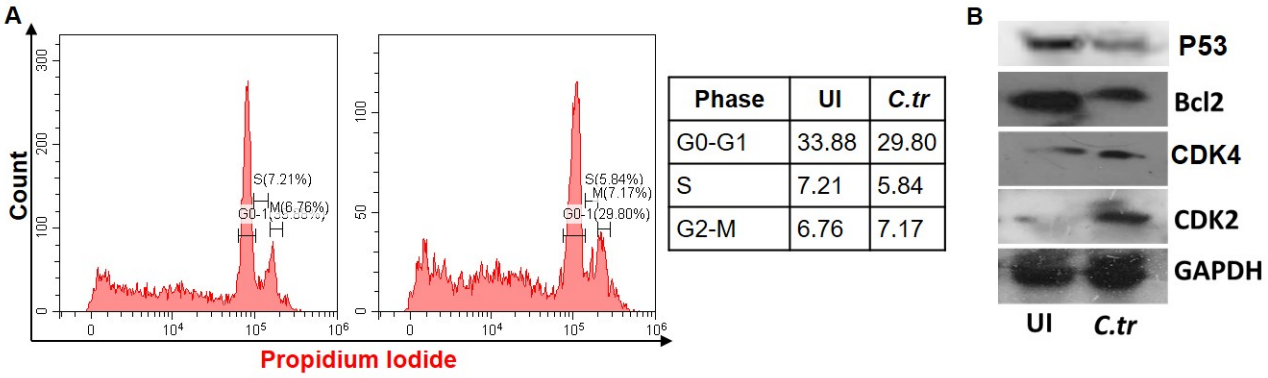


Figure 1.15: Chronic and secondary *C. trachomatis* infection increases Treg in the inguinal lymph nodes of infected mice. Inguinal lymph nodes were collected and single-cell suspensions were prepared. $CD4^+$ $Foxp3^+$ cells were gated and their IL10 and TGFβ expression were dot plotted (A). % of Treg in $CD4^+$ T cells were plotted (B)($CD25^+$ $Foxp3^+$ vs $CD25^+$ $Foxp3^+$-*; $CD25^+$ $Foxp3^+$ vs $CD25^+$ $Foxp3^+$ IL-10^+-#; $CD25^+$ $Foxp3^+$ IL-10^+ TGFβ^+ vs $CD25^+$ $Foxp3^+$ IL-10^+ TGFβ^+-†). Histogram representing the IL10 and TGFβ expression in $CD4^+$ T cells (C). Hierarchical Sunburst graphs of the mean values obtained from the 1.14 and 1.15 experiment (D). (n=4; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

survival and death pathways. The recurrent infection is shown to cause immune suppression which is explained in detail in the previous section.



Phase	UI	*C.tr*
G0-G1	33.88	29.80
S	7.21	5.84
G2-M	6.76	7.17

Figure 1.16: *C.trachomatis* alters cell cycle. Mouse cervical epithelial cells were obtained from BALB/c mice genital tract. Epithelial cells were infected with *Chlamydia* and analyzed for cell cycle by PI staining using a flow cytometer (A). In parallel experiments performed with 10^6 epithelial cells, cells were collected after 24hrs of infection and analyzed for cell cycle proteins by Immunoblotting

To delineate the molecular mechanism, epithelial cells were infected with *C.trachomatis* and analyzed for their cell cycle. *C. trachomatis* infected epithelial cells show an altered cell cycle compared to control cells. The infection promotes the progression of cells to the M phase driving cellular proliferation. The change in the cellular process could be mediated by altering proteins involved in the cell cycle. *C. trachomatis* infection induced the expression of cyclin-dependent kinases, CDK4 and CDK6 in the epithelial cells which facilitate the transition of cell cycle phases. Infected cells also show a decrease in the cell cycle regulator, p53, and apoptotic control protein Bcl2. These changes brought by the *C. trachomatis* infection together might induce cellular proliferation or resist death. Concurrent to our observation, previously, E Gonzalez et al., [42] have reported that *C. trachomatis* manipulates MDM2—P53 axis. These proteins we have reported are under the regulation of MDM2—P53 axis, thus alteration in this pathway might lead to cellular changes.

Chronic *C. trachomatis* genital infection resulted in decreased p53 levels in the genital tract as shown by immunofluorescence(Fig. A) and Western blot (Fig. C). This was accompanied by an increase in cellular proliferation in the epithelial region of the cervical tissue. The increase in Ki67 indicates that proliferation in the cervical tissue of the infected mice has increased compared to control mice (Fig. B). The tissue collected from the infected mice also show increased expression of CD133, a stem cell marker (Fig. C). As shown in the results (Fig.1.16) of epithelial cells, *C. trachomatis* infected mice also show decreased CDK4, CDK6 proteins and BCL2 proteins.

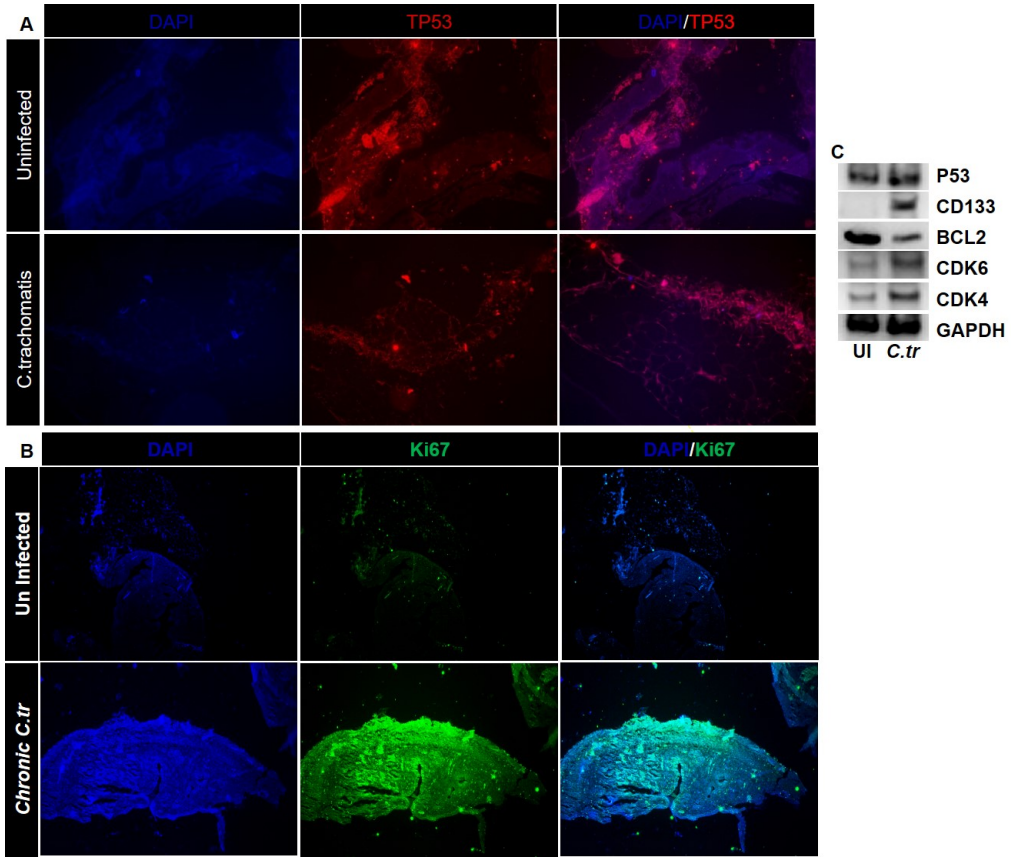


Figure 1.17: *C.trachomatis* genital infection induces proliferation. Balb/c mice were infected repeatedly for 20 days and analysed for P53 expression (A) and Ki67 (B) expression in the cervix. Cervical tissue was collected and protein was extracted in RIPA buffer. cell cycle proteins expression were analyzed by Western blot (C).(n=3)

To understand the molecular mechanism of the changes bought by the co-infection, we have done in silico meta-analysis with *C. trachomatis* infected and HPV-infected samples. The top 250 genes from the multiple studies conducted with HPV genes were pooled to observe HPV-manipulated genes. Simultaneously, studies with genes manipulated during *C. trachomatis* infections were curated. These two groups of genes manipulated by the pathogens were combined, as during co-infection individually these pathogens might be manipulating these genes. These genes converged along with their interacting proteins as reported earlier. *C.trachomatis* with their inclusion proteins and others interacts with many host proteins interfering with their functions. HPV proteins are also known to interact with many host proteins with their E and L proteins.

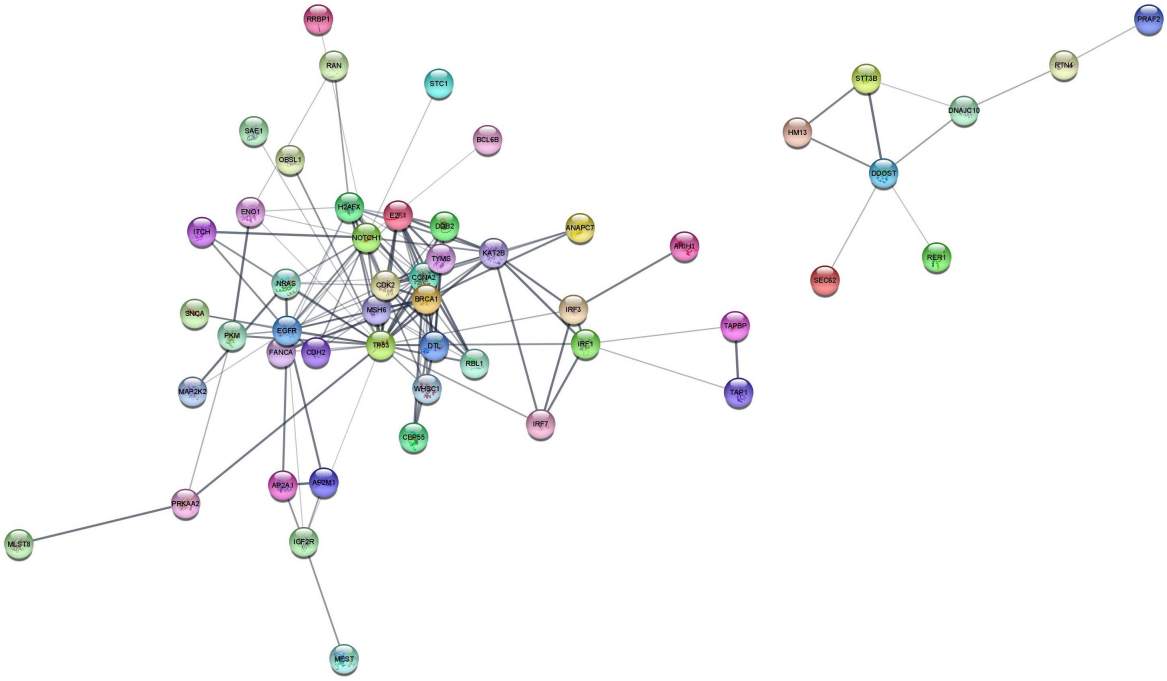


Figure 1.18: String analysis of the *C. trachomatis* and HPV commonly manipulated genes. The proteins are represented in **??**

These network of interactions that could possibly be happening during the co-infection are shown in the figure. HPV is a well know etiological agent in cervical cancer. Even though *Chlamydia* is known to occur in many cervical cancer patients, the molecular basis for the tumor formation or progression has not been understood. Some of the genes manipulated and are involved in host-pathogen interaction in chlamydial infection are well-reported in cervical cancer. This indicates that *C trachomatis* infection might increase the manipulation of these genes worsening the pathological condition. The genes which are commonly manipulated by both the HPV and *C trachomatis* and their relation to cervical cancer are reported in the below table**??**.

1.3.6 *C. trachomatis* and HPV co-infection induce tumorigenic changes in mouse cervical tissue

C. trachomatis manipulates cell survival and cell death pathways as reported earlier by many scientists and by us in previous results. However, the co-infection has not been experimentally proved in an in vivo model. We have transfected mouse genital tract with E6-E7 overexpressing plasmid and infected repeatedly with *C. trachomatis* as mentioned in M & M. The infection was able to induce IL-6 and TGFβ which are often reported to act as oncogenic cytokines in tumor progression. These cytokines were further increased in presence of E6-E7 oncogenes. Mice of the co-infection groups show

increased CDK2 and CDK6 proteins indicating an alteration in cell cycle progression. Further, the mice expressed lower levels of Bcl2 and p53 proteins in the cervical tissue thus decreasing the apoptosis in the tissue. Cervical tissue also increased expression of CD133 indicating the presence of stem-like cells in the cervical tissue.

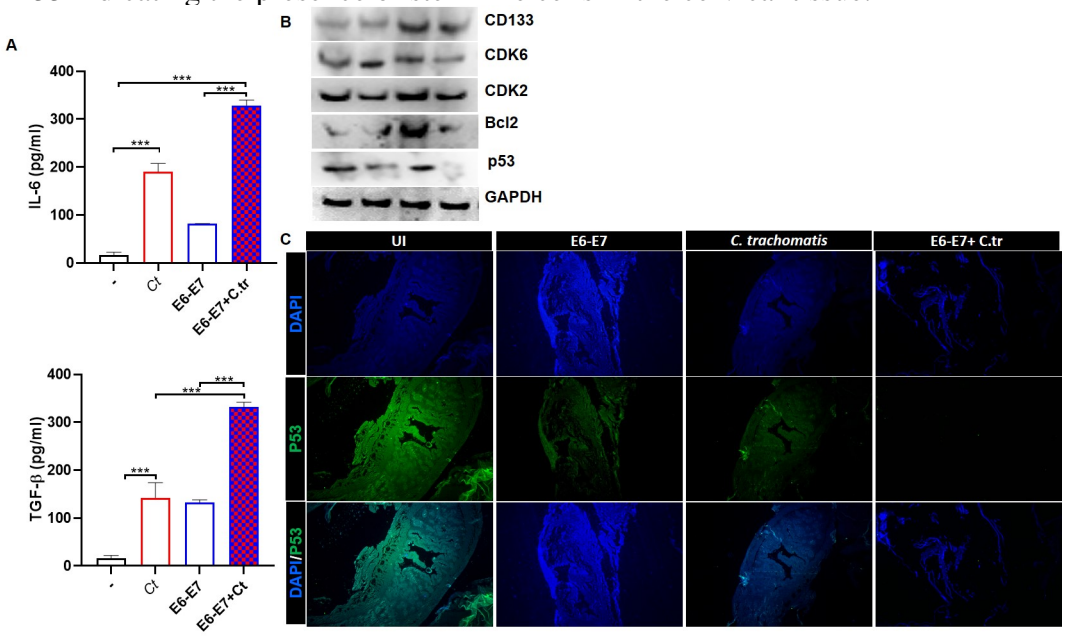


Figure 1.19: HPV and *Chlamydia* induces immunesupression and alters cell cycle protens in mice. Mice were either genitally transfected with E6-E7 or infected with *Chlamydia* or both and analysed molecular markers. Serum cytokines were measured by ELISA (A). Cervical tissue was lysed to extract protein and analyse cell cycle markers by Western blotting (B). From a parallel experiment, cervical tissue was collected in 4%PFA and sections were analysed for p53 expression by immunohistochemistry. (n=3; ***indicates ρ<0.0001;**indicates ρ<0.005;**indicates ρ<0.01)

1.4 Conclusion

The thesis provides the following evidence through which we were able to prove that *C. trachomatis* induces immune suppression which in the presence or absence of HPV contributes to oncogenic changes in cervical epithelial cells.

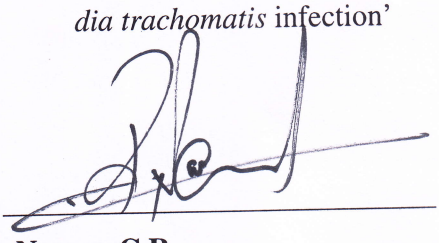
1. *C. trachomatis* induces M_2 polarisation in macrophages
2. Chronic *C. trachomatis* facilitates increased M_2 macrophages and MDSC differentiation
3. *C. trachomatis* decreased CD40 expression and tilts its signalling towards IL10 secretion
4. *Chronic C. trachomatis* impairs INFγ secretion from T cells and decreases sensitivity to INFγ in macrophages

5. *C. trachomatis* alters host cell cycle and resist cell death

6. Presence of HPV 16 E6-E7 proteins exaggerates *C. trachomatis* mediated oncogenic traits

Submitted in Journal(s) / Under Preparation

- Challagundla, N., Shah, D., and Rajput, RA. (2022), 'IFNγ insufficiency during mouse intravaginal *Chlamydia trachomatis* infection exacerbates alternative activation in macrophages with compromised CD40 functions'. doi:10.1101/2022.07.05.497583.
- Challagundla, N., Phadnis, D., Gupta, A., and Rajput, RA. (2022), 'Lipid manipulation by intracellular bacteria-Moonlighting for immune evasion'.
- Challagundla, N., Yadav, S., Vora, H., and Rajput, RA. (2022), 'Differential myelopoiesis in susceptible BALB/c and resistant C57BL/6 during chronic *Chlamydia trachomatis* infection'



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List of Publications

Research and Review articles

- Patel, D., Gaikwad, S., Challagundla, N., Nivsarkar, M., and Rajput, RA. (2018), 'Spleen tyrosine kinase inhibition ameliorates airway inflammation through modulation of NLRP3 inflammasome and Th17/Treg axis', *International immunopharmacology*, 54, 375–384. doi:10.1016/j.intimp.2017.11.026.

- Challagundla, N., Rokkam, P., Zala, D., R, R., and Rajput, RA. (2020), 'Chlamydia trachomatis alters p53 in epithelial cells and manipulates host cell lipid metabolism and CD40 signalling polarizing anti-inflammatory macrophages', *Molecular Biology of the Cell*, 31 (26) (abstract#P260). doi:10.1091/mbc.E20-10-0665.

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Conference Proceedings

Oral Presentations

- Challagundla, N., and Rajput, RA. (2022), 'Chronic *Chlamydia trachomatis* intravaginal infection induces skewed myelopoiesis towards M_2 polarization decapacitating T cells at cervix hindering IFNγ secretion', *49th Annual conference of Immunological society of India (Immunocon 2022)* **nominated for G.P.Talwar Young Scientist Award**.

- Challagundla, N., Phadnis, D., Gupta, A., and Rajput, RA. (2021), '*Chlamydia trachomatis* manipulates plasma membrane cholesterol and ceramide levels to manipulate CD40 mediated signaling', *48th Annual conference of Immunological society of India (Immunocon 2021)* **Best oral presentation Award**.

- Challagundla, N., Rokkam, P., Zala, D., R, R., and Rajput, RA. (2020), '*Chlamydia trachomatis* manipulates CD40 decapitating T cell activation as a pro-survival strategy', *IAR conclave* **Best oral presentation Award**.

- Challagundla, N., and Rajput, RA. (2018), '*Chlamydia trachomatis* manipulates host cell immune response via generation of anti-inflammatory macrophages leading to persistent infection', *IUIS-IIS-FIMSA Course work*

Poster Presentations

- Challagundla, N., Rokkam, P., Zala, D., R, R., and Rajput, RA. (2020), '*Chlamydia trachomatis* alters CD40 decapitating T cell activation as a pro-survival strategy', *IAR conclave*

- Challagundla, N., and Rajput, RA. (2018), '*Chlamydia trachomatis* manipulates CD40-inflammasome axis to modulate the immune response and generates pro-tumor conditions', *45th Annual conference of Immunological society of India (Immunocon 2018)*

Submitted in Journal(s) / Under Preparation

- Challagundla, N., Shah, D., and Rajput, RA. (2022), 'IFNγ insufficiency during mouse intravaginal *Chlamydia trachomatis* infection exacerbates alternative activation in macrophages with compromised CD40 functions'. doi:10.1101/2022.07.05.497583.
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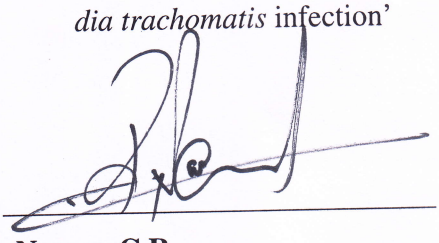

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